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Report

The Habenula Prevents Helpless Behavior in Larval Zebrafish

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Summary

Animals quickly learn to avoid predictable danger. However, if pre-exposed to a strong stressor, they do not display avoidance even if this causes continued contact with painful stimuli [1, 2]. In rodents, lesioning the habenula, an epithalamic structure that regulates the monoaminergic system, has been reported to reduce avoidance deficits caused by inescapable shock [3]. This is consistent with findings that inability to overcome a stressor is accompanied by an increase in serotonin levels [4]. However, other studies conclude that habenula lesions cause avoidance deficits [5, 6]. These contradictory results may be caused by lesions affecting unintended regions [6]. To clarify the role of the habenula, we used larval zebrafish, whose transparency and amenability to genetic manipulation enables more precise disruption of cells. We show that larval zebrafish learn to avoid a light that has been paired with a mild shock but fail to do so when pre-exposed to inescapable shock. Photobleaching of habenula afferents expressing the photosensitizer KillerRed causes a similar failure in avoidance. Expression of tetanus toxin in dorsal habenula neurons is sufficient to prevent avoidance. We suggest that this region may signal the ability to control a stressor, and that its disruption could contribute to anxiety disorders.

Results

Larval Zebrafish Learn Avoidance

Fish were placed in a rectangular shuttle box that had an electrode pair and a red light-emitting diode (LED) at each end (Figure 1A). The paired escapable shock paradigm comprised ten consecutive trials of a 5 s red light (conditioned stimulus; CS) that coterminated with a 100 ms electric shock (unconditioned stimulus; US) (Figure 1B). In each trial, the CS and US were presented on one side of the shuttle box only, with side determined by fish position at the scheduled time of CS presentation. The eleventh trial was a probe trial in which only the CS was presented for 5 s. As controls, fish were exposed to ten trials of unpaired stimuli or to CS alone (Figure 1B).

Fish were assessed for their ability to learn to avoid the shock by moving away from the illuminated LED and crossing the virtual midline of the tank, within 5 s of CS onset. Only CS-US paired fish displayed this response in the probe trial [Pearson $\chi^2(2, n = 30) = 18.10$, $p < 0.001$, Cramer's $V = 0.777$; Figure 1C]. Midline crossing was accompanied by an increase in swimming speed (Figure 1D) during the final second of CS presentation [$\chi^2(2, n = 30) = 11.78$, $p = 0.003$, $\eta^2 = 0.41$, Kruskal-Wallis test]. When exposed to an inescapable shock prior to conditioning, fish did not cross the midline in the probe trial, in contrast to fish without preshock [Pearson $\chi^2(1, n = 20) = 13.33$, $p < 0.001$, $\phi = 0.816$; Figure 1C]. This can be seen in the trajectory plotted in Figure 1E. Preshocked fish reduced swim speed until CS offset, in contrast to nonshocked fish [$\chi^2(1, n = 20) = 12.62$, $p < 0.001$, $\eta^2 = 0.66$, Kruskal-Wallis test; Figure 1F]. The ability of larval fish to actively avoid the shock is thus prevented by pre-exposure to an uncontrollable stressor.

Optical Disruption of Habenula Afferents Inhibits Avoidance Learning

To disrupt neural circuits involving the habenula, we used the fact that the line tested here (KR11) contains membrane-targeted KillerRed—a photosensitizer [7]—in habenula afferents from the ventrolateral forebrain (Figures 2A–2C; see also Movie S1 available online). To characterize these neurons, we examined markers that are found in different subsets of habenula afferents in mammals. Calretinin and calbindin were detected in neurons projecting to restricted neuropils (Figures 2D–2F; Figure S1), whereas GABA and GAD65/67 were expressed in very few neurons (Figures 2G and 2H). Somatostatin was not coexpressed (Figure 2I) but was found in adjacent cells (Figure S1D).

KillerRed releases reactive oxygen species when illuminated with green light [8]. Photobleaching of KillerRed-expressing zebrafish neurons resulted in specific binding of annexin V, which is an indication of membrane damage [9]. Labeling occurred rapidly (Figure S2), persisted for at least up to 6 hr—the period of conditioning and testing—and was restricted to KillerRed-expressing cells (Figures 3A and 3B). No uptake of acridine orange, which would occur if the cells were dying, was detected in habenula afferents during this time (Figure 3C). KillerRed fluorescence recovered gradually, appearing dimly in axons innervating the habenula within one day (Figure 3D). These results suggest that photobleaching, under illumination conditions used here, does not kill KillerRed-expressing cells in the KR11 line but causes damage.

When KR11 fish with photobleached habenula afferents were conditioned in the shuttle box, they did not display the avoidance response in the probe trial, in contrast to controls [Pearson $\chi^2(2, n = 30) = 14.12$, $p = 0.001$, Cramer's $V = 0.686$; Figure 3E; Movie S2; Movie S3]. There was also reduced mobility before CS offset, compared to nonirradiated siblings [$\chi^2(1, n = 20) = 14.27$, $p < 0.001$, $\eta^2 = 0.75$, Kruskal-Wallis test; Figure 3F]. This deficit is unlikely to be caused by nonspecific effects of photobleaching because photobleaching of

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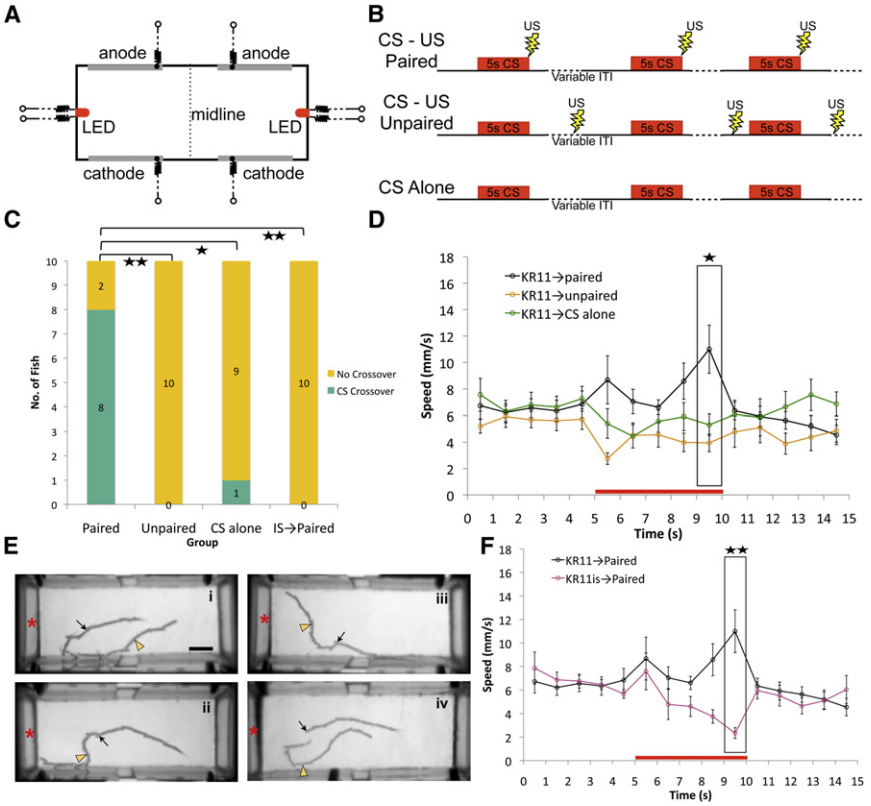

Figure 1. Avoidance in Larval Zebrafish Is Affected by Pre-exposure to Inescapable Shock

(A) Diagram of the apparatus used for conditioning.

(B) Regimens for paired conditioned stimulus (CS)-unconditioned stimulus (US), unpaired CS-US, and CS-alone conditioning.

(C) Movement of fish across the midline prior to CS offset in the probe trial.

(D) Mean swim speed of fish in response to CS in the probe trial. The red bar indicates presence of CS. Swim speeds during the final second of CS presentation (black box) were subjected to between-group statistical analysis using the Kruskal-Wallis test. Prior to analysis, swim speeds of individual fish were corrected for possible baseline differences by subtracting the swim speed during the 1 s immediately preceding CS onset from the swim speed during the final second of the CS.

(Ei–Eiv) Trajectories of fish in the probe trial. Black arrow indicates CS onset; yellow arrowhead indicates offset. Red asterisk indicates position of LED. Scale bar represents 1 cm at midlevel of chamber.

(Ei) Paired.

(Eii) Unpaired.

(Eiii) CS alone.

(Eiv) Preshocked, then paired.

(F) Mean swim speed of fish exposed to inescapable shock prior to conditioning (pink line), compared with fish that had not been preshocked (black line).

The following abbreviations are also used: ITI, intertrial interval; IS, inescapable shock. Error bars indicate standard error of the mean (SEM). **$p < 0.001$; *$p < 0.05$. All fish are KR11.

KillerRed-expressing cells close to the habenula in another transgenic line, KR4 (Figure S2), did not prevent avoidance learning [$\chi^2(2, n = 30) = 16.73$, $p < 0.001$, $\eta^2 = 0.58$, Kruskal-Wallis test; $z = -3.78$; $p_{pw} < 0.001$ ($\alpha_{pw} = 0.017$), Mann-Whitney U test; Figures 3E and 3G]. Disruption of habenula afferents appears to prevent acquisition, rather than expression, of the avoidance response because photobleaching after conditioning did not affect avoidance [$z = -3.78$; $p_{pw} = 0.004$ ($\alpha_{pw} = 0.025$), Mann-Whitney U test; Figures 3E and 3G]. Many photobleached KR11 fish displayed avoidance at trial 2, but few did so at later trials (Figure 3H). This is similar to fish exposed to inescapable shock before conditioning and is reminiscent of the original experiment by Overmeier and Seligman [1], where dogs that initially made an avoidance response failed to do so subsequently.

Many KR11 fish that had been photobleached and subjected to shock displayed a startle response after onset of light in the probe trial [Pearson $\chi^2(7, n = 80) = 28.80$, $p < 0.001$, Cramer's $V = 0.60$; Figures 3I and 3J]. Startle, which is an indicator of anxiety [10], was seen less often in nonphotobleached fish, and never in fish that had not received a shock. Startle was also never seen in nonphotobleached fish exposed to US alone, which received the maximum number of shocks, indicating that startle was not due to increased exposure to shock. A two-way contingency table analysis was conducted to evaluate differences in startle when shock was applied to photobleached or nonphotobleached fish. A significant relationship between irradiation and startle was found [Pearson $\chi^2(1, n = 60) = 14.70$, $p < 0.001$, $\phi = 0.495$; Figure 3J]; the probability of a fish displaying startle in response to light was ~5.67 times higher when the fish had been photobleached. Photobleached KR11 fish developed a startle response to the CS even when CS and US were explicitly unpaired or when only the US was presented. This may reflect increased contextual conditioning, which is known to accompany increased anxiety [11].

Expression of Tetanus Toxin in Dorsal Habenula Efferents Inhibits Active Avoidance

We further tested the involvement of the habenula by using the GAL4/UAS system to express the light chain of tetanus toxin (TeTXlc), which silences neurons by cleaving synaptobrevin [12], in habenula neurons. We used the GAL4^{s1019t} enhancer trap line, which drives UAS:Kaede expression strongly in the dorsal habenula [13] (Figures 4A and 4B; Figures S3A and S3B). These were crossed to fish carrying UAS:TeTXlc-CFP [14].

To verify that TeTXlc was expressed, we labeled the fish with an antibody that recognizes the CFP tag. TeTXlc-CFP was detected in neurons located in the medial regions of the dorsal habenula (Figure 4C) that mostly innervate a single neuropil (Figures S3A–S3D; Movie S4), whereas Kaede was mainly expressed in nonoverlapping neurons of the dorsal habenula (Figure 4C; Figure S3; Movie S4); this reflects the variegation of some transgenes in zebrafish [15]. Some TeTXlc-CFP expressing neurons were detected elsewhere in the brain in six fish analyzed after conditioning (Figures S3E–S3H). However, their location differed from fish to fish, and consistent expression was seen only in the dorsal habenula.

All GAL4^{s1019t}/UAS:TeTXlc-CFP fish were deficient in avoidance in the probe trial, in contrast to siblings that did not

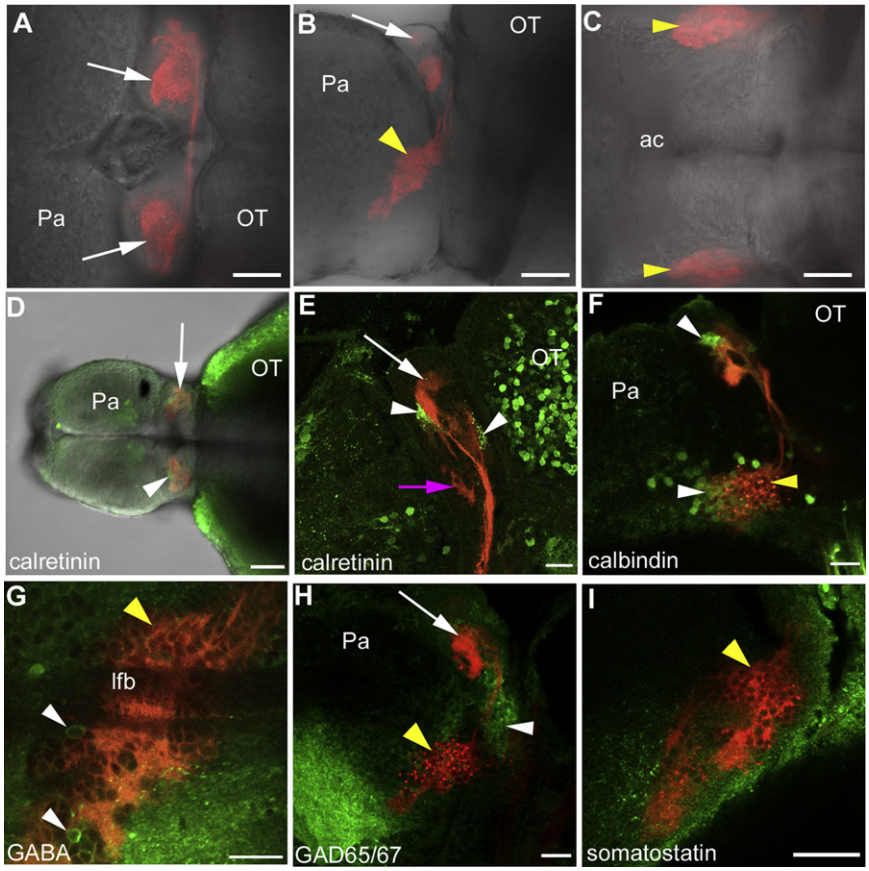

Figure 2. Characterization of KillerRed-Expressing Habenula Afferents

(A–C) Forebrain of a KR11 zebrafish in dorsal (A), lateral (B), and ventral (C) views. KillerRed is expressed in axons that innervate the habenula (arrows). Cell bodies of KillerRed-expressing neurons (arrowheads) are in the ventral forebrain (B), in a lateral position (C).

(D) Dorsal view of a 2-week-old fish showing calretinin label in restricted habenula neuropils (arrowhead).

(E) Sagittal section showing calretinin label in two dorsal habenula neuropils (arrowheads).

(F) Lateral view (projection) showing calbindin label in KillerRed-positive neurons (white arrowhead).

(G) Lateral view showing rare GABA-positive neurons (arrowheads) in the KillerRed-expressing population. The lateral forebrain bundle is visible in this optical section.

(H) Projection of the left side, showing GAD65/67 label in neurons (white arrowhead) dorsal to the KillerRed cluster.

(I) Optical section, lateral view, showing lack of somatostatin label in neurons expressing KillerRed.

The following abbreviations are used: Pa, pallium; OT, optic tectum; ac, anterior commissure; lfb, lateral forebrain bundle. Scale bars represent 50 μm in (A)–(D) and 20 μm in (E)–(I). Yellow arrowheads indicate KillerRed-expressing cells; white arrows indicate the habenula; pink arrow indicates ventral habenula. Anterior is to the left in all cases. Fish in (F)–(H) are 3 weeks old; fish in (A)–(C), (E), and (I) are 4 weeks old.

express TeTXlc-CFP or UAS:TeTXlc-CFP fish that did not carry the GAL4 driver [Pearson $\chi^2(2, n = 30) = 17.14$, $p < 0.001$, Cramer's $V = 0.756$; $\chi^2(2, n = 30) = 14.21$, $p = 0.001$, $\eta^2 = 0.49$, Kruskal-Wallis test; Figures 4D and 4E]. A deficit in avoidance was not seen in early trials (Figure 4F). Hence, when TeTXlc-CFP is expressed in dorsal habenula neurons, larval zebrafish behave as though they had been subjected previously to inescapable shock or to disruption of habenula afferents, tolerating shock even though avoidance is an option.

Discussion

Disrupting neural circuitry involving the habenula, via two different methods, caused a deficit in active avoidance in larval zebrafish. Expression of the light chain of tetanus toxin is a well-established method to inhibit neural firing, having been used in mice [16], *Drosophila* [17], and zebrafish [14]. Photobleaching of membrane-targeted KillerRed has been used to kill HeLa cells in vitro [7], but cell-type variation in killing efficiency has been reported [18], and no significant killing of zebrafish neurons was seen in the KR11 line with the irradiation conditions used here. Nevertheless, photobleaching of KillerRed-expressing habenula afferents appears to have a specific effect. First, annexin V binds only to these neurons after irradiation. Although annexin V is commonly used for detecting apoptosis because of its ability to bind phosphatidylserine, it also binds to malondialdehyde [9], a product of lipid peroxidation. Lipid peroxidation does not directly kill cells but disrupts membrane proteins [19] and reduces synaptic efficacy and action potential generation in mammalian neurons [20, 21]. A similar phenomenon may occur in zebrafish habenula afferents, although we cannot exclude other effects. Second, when KillerRed-expressing habenula afferents were irradiated, a change in behavior was seen. This change was specific to irradiation of these neurons prior to conditioning and did not occur when fish expressing KillerRed in nearby cells were irradiated.

Based on efferent connectivity, the dorsal and ventral subnuclei of the zebrafish habenula have clear homology to the mammalian medial and lateral habenula, respectively [22]. The mammalian medial habenula receives substantial input from posterior septal neurons that are largely calretinin and calbindin positive [23], whereas the lateral habenula receives input primarily from the entopeduncular nucleus (EN), which is calbindin negative [24] and in part GABAergic [25]; septal neurons also innervate the medial portion of the lateral habenula [26]. Given the lateral position of some septal neurons in the zebrafish brain [27, 28], it is possible that a number of KillerRed-expressing neurons, particularly those that express calretinin or calbindin and innervate dorsal nuclei, are homologs of posterior septal neurons such as those in the bed nucleus of the stria medullaris [27]. We were unable to confirm whether the cluster includes the EN. Somatostatin, which is expressed in the EN in mammals [29] and may be a marker of this structure in teleosts [30, 31] (but see [32]), could not be detected.

Amat et al. have proposed that the ability to control an outcome actively inhibits stress-induced neural activity [33]. Specifically, they suggest that in mammals, the ventral medial prefrontal cortex detects the ability of a particular behavior to

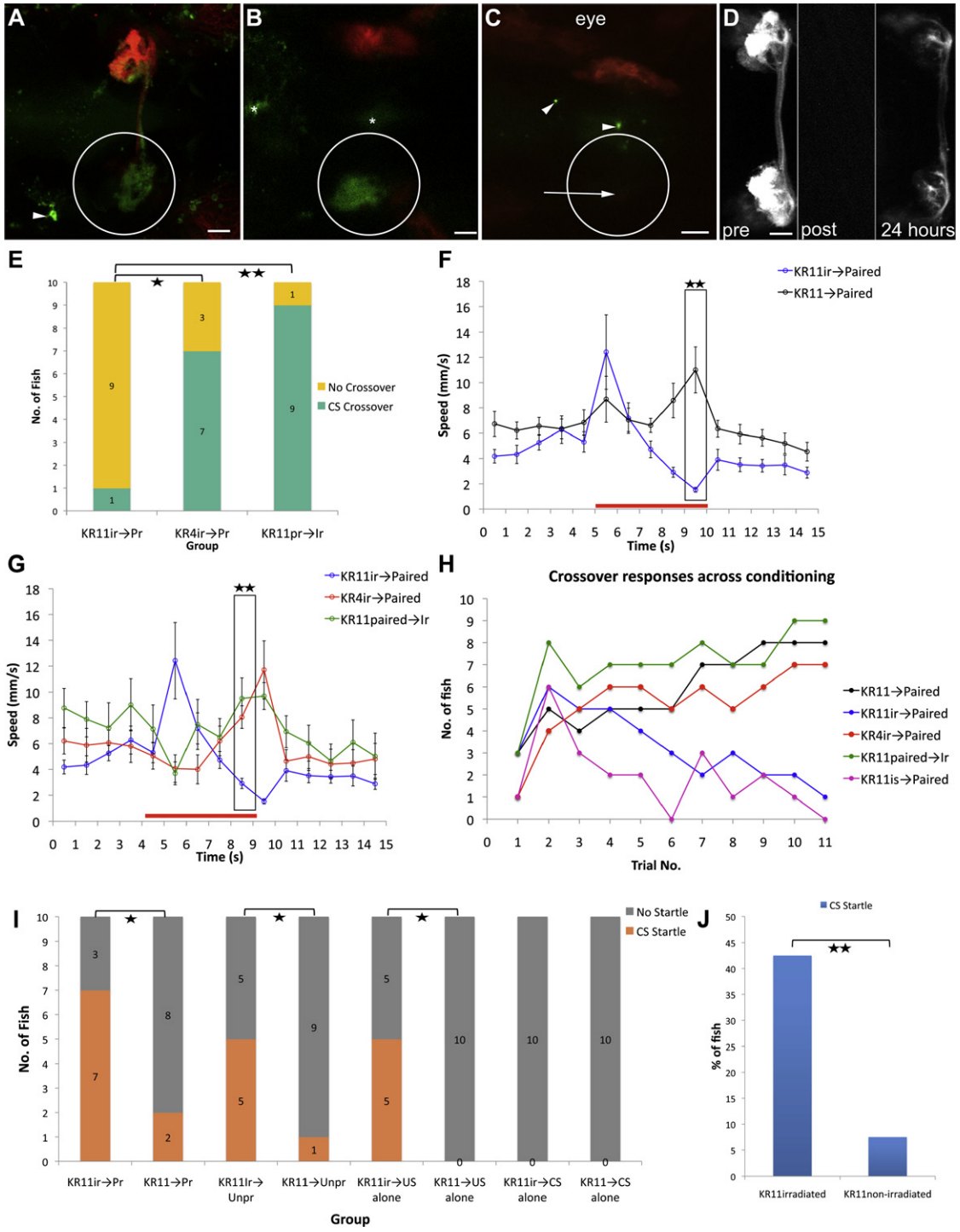

Figure 3. Effect of Photobleaching KillerRed-Expressing Neurons

(A) FITC-annexin V label in a KR11 fish 3 hr after photobleaching of the left habenula, in the region marked by the white circle. Annexin V binds only to habenula afferents and not to efferents. Label is visible in axons that terminate in the contralateral habenula, but not in axons that originate from that (nonirradiated) side. One cell (arrowhead), presumably undergoing apoptosis, is labeled outside the irradiated region.

(B) Deeper focus of the same fish, showing FITC-annexin V label of the cell bodies in the side that was irradiated. Asterisks indicate sites of FITC-annexin V injection.

(C) A fish 5 hr after irradiation of the left habenula. A few cells have taken up acridine orange (arrowheads), but these are not located in the region of cells that had been expressing KillerRed (arrow).

(D) Fluorescence recovery of KillerRed after photobleaching.

(E) The effect of photobleaching KillerRed-expressing cells on the avoidance response.

(F) Mean swim speed in the probe trial for KR11 fish photobleached before conditioning, compared with nonphotobleached fish.

(G) Mean swim speed in the probe trial of photobleached KR4 and KR11 fish.

(H) Number of fish showing avoidance, as a function of trial number.

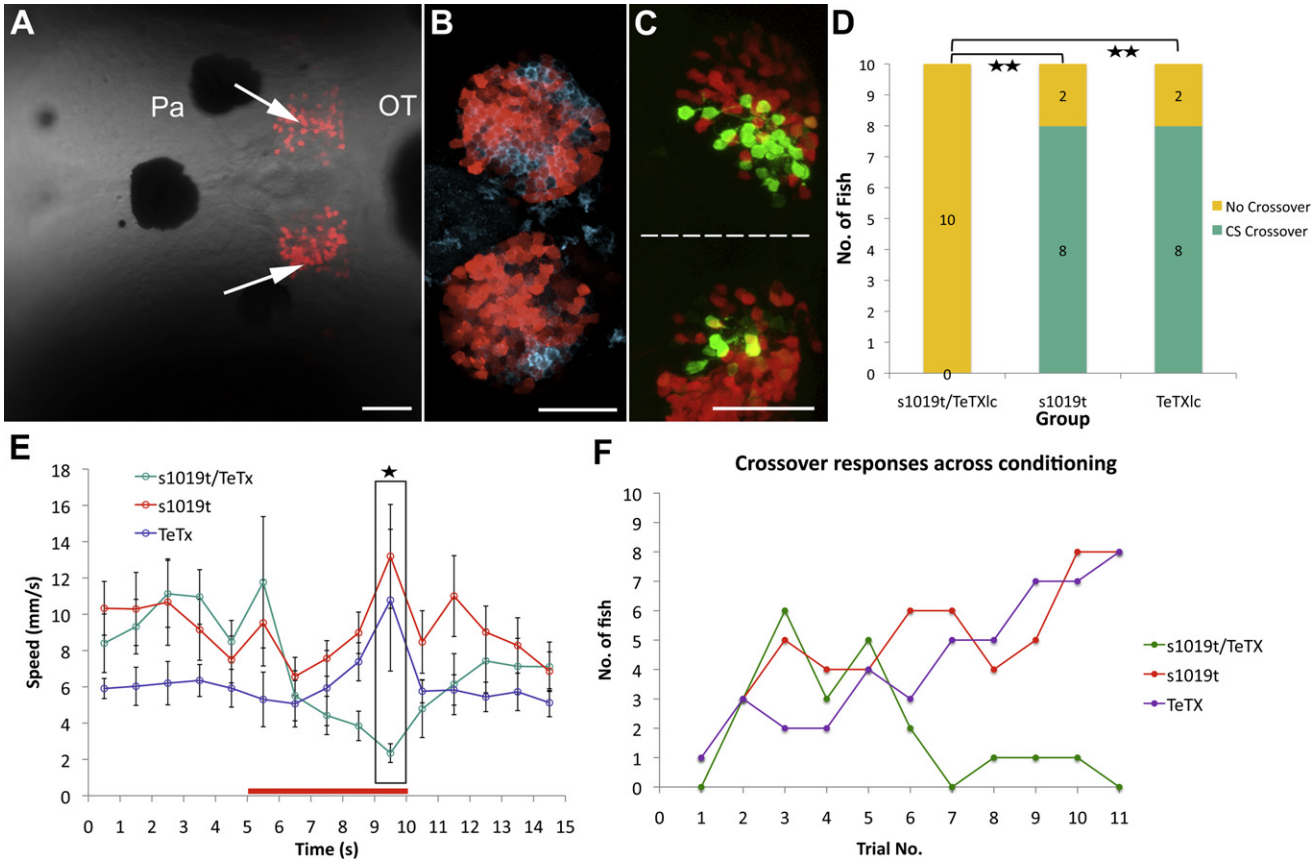

Figure 4. Expression of the Light Chain of Tetanus Toxin in Dorsal Habenula Neurons Prevents Avoidance Learning

(A) Kaede expression in habenula neurons (arrows) of an 18-day-old GAL4^{s1019t}/UAS:Kaede fish. This image is a projection of optical sections spanning 90 μm.

(B) Expression of Kaede (red) relative to Ron [37] (cyan), a protein found in the dorsal habenula. This image is a projection spanning 18 μm.

(C) A 3-week-old triple transgenic (GAL4^{s1019t}/UAS:Kaede/UAS:TeTXlc-CFP). Habenula neurons expressing TeTXlc-CFP (green) are in the medial regions. There is incomplete overlap with Kaede (red) expression.

(D and E) Avoidance response (D) and mean swim speed (E) in the probe trial following conditioning with paired CS and US for fish carrying GAL4^{s1019t}/UAS:Kaede/UAS:TeTXlc-CFP, GAL4^{s1019t}/UAS:Kaede, or UAS:TeTXlc-CFP.

(F) Avoidance response as a function of trial number.

Fish are shown in dorsal view; anterior to the left. Scale bars represents 50 μm. s1019t refers to the GAL4^{s1019t} line. The dotted line in (C) is the midline. Error bars indicate SEM. **p < 0.001; *p < 0.05.

overcome a stressor and regulates the dorsal raphe nucleus, thereby preventing the behavioral sequelae of uncontrollable stress. One interpretation of the present results, supported by a recent finding that adult fish with TeTXlc in the dorsal habenula also show increased freezing rather than escape during conditioning [34], is that the dorsal habenula is a part of the circuit that evaluates control over stressors in zebrafish; one function may be to inhibit anxiety following a successful response to a threat. This would be consistent with the loss of avoidance after several trials and the many reports that habenula lesions show an effect only in stressful situations [5, 35, 36]. Based on the suggestion of Wilcox et al. [6] that the medial habenula is required for avoidance learning in rodents, this function may be evolutionarily conserved in vertebrates. A prediction from the results presented here is that disruption of the medial habenula may underlie some mental disorders that are characterized by uncontrollable anxiety and helplessness.

Supplemental Information

Supplemental Information includes three figures, four movies, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.cub.2010.11.025.

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(I) Number of fish displaying a startle response.

(J) Percentage of startle displayed in the probe trial by fish that had been subjected to US during conditioning.

All micrographs are dorsal views, with anterior to the left. Fish in (A)–(D) are 3 weeks old. Scale bars represent 20 μm in (A), (B), and (D) and 50 μm in (C). The following abbreviations are used: ir, irradiated; Pr, paired. Error bars indicate SEM. **p < 0.001; *p < 0.05.

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